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Increased Sensitivity to Staphylococcal Enterotoxin B following Adenoviral Infection

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Staphylococcal enterotoxin B induces toxic shock and is a major virulence factor of staphylococcal diseases. We examined the effects of systemic adenoviral infection on responses to staphylococcal enterotoxin B in a murine model. We found that adenoviral infection markedly increases the severity of liver injury following exposure to staphylococcal enterotoxin B without D-galactosamine sensitization. In adenovirus-infected mice, staphylococcal enterotoxin B triggered a more profound hypothermia and increased apoptosis in the liver. Consistent with these observations, we also found that adenoviral infection primed for an increased production of gamma interferon in vivo and in vitro following stimulation with staphylococcal enterotoxin B. Gamma-interferon-knockout mice did not show increased sensitivity to staphylococcal enterotoxin B following adenoviral infection. These data suggest that a preexisting viral infection primes mice for subsequent staphylococcal enterotoxin B exposure, possibly via a gamma-interferon-mediated mechanism.

Staphylococcal enterotoxin B (SEB) belongs to a large family of staphylococcal and streptococcal pyrogenic toxic superantigens and is a major virulence factor of staphylococcal diseases (7). SEB induces toxic shock syndrome and severe food poisoning in humans (7). Aerosolized SEB has also been shown to induce a fatal respiratory distress syndrome in nonhuman primates and is considered a potential threat to public health and safety (31).

As a superantigen, SEB binds to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and to specific T-cell receptor (TCR) Vβ chains (29). This interaction results in activation of a large proportion of T cells (up to 30%) and massive release of proinflammatory cytokines (4). In laboratory mice, SEB binds to MHC class II molecules I-A and I-E and activates almost all T cells bearing TCR Vβ3, -7, -8.1, -8.2, -8.3, and -17 (29). Mice are known to have natural resistance to the toxic effects of SEB due to the lower affinity of SEB for mouse MHC class II molecules than for human MHC class II molecules (29). Many studies have used D-galactosamine (D-Gal), a hepatocyte-specific inhibitor of transcription, to sensitize mice to the lethal effect of SEB (30, 32). Early release of tumor necrosis factor alpha (TNF-α) by activated T cells and TNF-α-induced hepatocyte apoptosis and liver failure have been shown to mediate the toxicity of SEB in this model (32). Production of high levels of gamma interferon (IFN-γ) following exposure to SEB has been well documented and contributes significantly to the pathogenesis of toxic shock (9, 30). On the other hand, interleukin 4 (IL-4), IL-6, and IL-10 have been shown to be protective in the mouse models of SEB-induced shock (2, 9, 30).

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SEB and toxic shock syndrome toxin 1 have been reported to be involved in the development of toxic shock as a complication of influenza virus infections (28). Multiple case reports have shown an association of toxic shock syndrome with prior viral infections (6, 43, 45). However, the mechanisms leading to such association have not been sufficiently investigated. A previous study demonstrated lethal synergism between influenza virus and SEB: mice infected with influenza virus for 7 days became susceptible to SEB-induced lethal shock (57). It was shown that TNF-α and IFN-γ were important, as neutralization of these cytokines protected against SEB-induced death. In another study, Vβ8.1 TCR-transgenic mice infected with lymphocytic choriomeningitis virus were also shown to become sensitive to SEB (42). In the latter study, neutralization of TNF-α provided partial protection against SEB, whereas neutralization of IFN-γ had no effect on survival. Both studies concluded that excessive activation of T cells was responsible for the increased sensitivity to SEB following prior exposure to virus.

Human adenoviruses are a common cause of respiratory, gastrointestinal, and eye infections (17). Generally, mild disease is observed in immunocompetent individuals; however, outbreaks of severe respiratory disease and pneumonia have been reported in young children and military recruits (3, 11, 39, 41). Adenoviruses are also known to increase the incidence and severity of secondary bacterial infections: associations of adenoviruses with bacterial pneumonia, otitis media, and conjunctivitis have been well documented in a number of epidemiological studies (23, 24, 38, 48). The mouse model of adenoviral infection has been extensively studied to evaluate the immune responses even though human adenoviruses replicate very poorly in mice (12). Robust cytotoxic lymphocyte-mediated responses to adenovirus are generated in mice despite the wide spectrum of the immune evasion mechanisms that prolong adenoviral persistence (34, 49). Systemic administration of adenoviral vectors results in effective transduction of hepa-

tocytes, which are subsequently eliminated by MHC class I-restricted $CD8^+$ T lymphocytes (51). NK cells and $CD4^+$ T cells also play important roles in the immune responses to adenoviruses (5, 36, 53).

Using a murine model of polymicrobial sepsis and adenoviral vectors, we have previously shown that systemic adenoviral infection increases the severity of secondary bacterial infections (8). These observations prompted us to determine whether altered responsiveness to bacterial exotoxins may mediate, in part, the increased sensitivity to secondary bacterial infections following exposure to adenovirus. We have recently reported that adenoviral infection induces TNF-α tolerance in mice and protects them against SEB-induced liver failure in the model with D-Gal treatment (56). It is recognized that the model with D-Gal treatment has certain limits: it is suitable only for estimating the sensitivity of the animals to TNF-α-mediated apoptosis of hepatocytes and liver failure and does not reproduce the complexity of events in toxic shock (1, 33). The outcome of exposure to SEB alone is not solely dependent on the level of TNF-α production (1). Thus, treatment with SEB alone is a better model for clinical implications of SEB exposure and allows analysis of a range of responses beyond TNF-α. The aim of the present study was to characterize the responses of adenovirus-infected mice to SEB without D-Gal sensitization. We hypothesized that adenoviral infection may sensitize mice to SEB due to generation of a strong Th1 response. We report here that adenoviral infection increases the severity of SEB-induced liver injury. The effect is due to excessive early production of IFN-γ by $CD4^+$ T cells and IFN-γ-mediated apoptosis in the liver. Our findings suggest that prior exposure to adenoviral infection may represent a serious risk factor for SEB-induced toxic shock.

MATERIALS AND METHODS

Adenoviral infection and SEB challenge. The animal use protocol was reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee. DBA/2 mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD) or from Harlan (Indianapolis, IN). IFN-γ-knockout mice [GKO; complete strain name C.129S7(B6)-*Ifng*^{tm1Ts}/J], backcrossed to a BALB/c background for more than 15 generations, and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice (females, 6 to 8 weeks old) were housed in a specific-pathogen-free environment for 7 to 10 days upon arrival. Human adenovirus serotype 5 was purchased from the American Type Culture Collection (VR-5; Manassas, VA). Adenoviral infections were established by intravenous injections of the adenovirus (10^6 PFU/mouse in 100 μl volume) to ketamine-xylazine-anesthetized mice. Control mice were anesthetized and received 100 μl of the carrier solution and were usually housed in the same biohazard containment rooms as the adenovirus-infected mice but kept in separate cages with top filters. Housing of control mice under the same conditions provided us with the confidence that the effects seen in adenovirus-infected mice were specific for adenovirus and did not come from exposure to other factors. SEB was purchased from Toxin Technologies Inc. (Sarasota, FL), reconstituted in pyrogen-free sterile phosphate-buffered saline, aliquoted, and stored at −20°C until use. Endotoxin contamination in the SEB preparations was in the range of 20 to 30 EU/mg. SEB challenge was performed by intraperitoneal injection using hypodermic needles. Control mice received an equivalent volume of phosphate-buffered saline. Mice were routinely monitored for the signs of distress for up to 24 h or until euthanized with an overdose of pentobarbital. During the course of the experiments, body temperature was measured using a veterinary infrared thermometer (Exergen, Watertown, MA). All experiments were started at the same time of day and performed in a temperature-controlled room. In some experiments, body temperature was also measured in control untreated mice in parallel with experimental groups to determine the effects of diurnal cycle. Following euthanasia, mice were exsanguinated by direct cardiac puncture to collect blood samples. Tissue samples were excised, snap-frozen in liquid nitrogen, and stored at −70°C until use.

Immunoblot analysis. Tissue samples were homogenized in ice-cold lysis buffer (0.05 M Tris, pH 7.4; 0.15 M NaCl; 1% NP-40) supplemented with 1× complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and 1× phosphatase inhibitors (Calbiochem, La Jolla, CA). Following sonication, tissue lysates were cleared by centrifugation at 16,000 × *g* for 10 min at 40°C. Protein concentrations in tissue lysates were measured using a Bradford assay (Bio-Rad, Hercules, CA) protein assay reagent. Western blot analysis was performed by separation of 50 μg/lane of proteins from individual lysates (two to four mice per group) in 7.5% sodium dodecyl sulfate-polyacrylamide gel and semidry transfer onto nitrocellulose (Hybond-ECL; Amersham, Arlington Heights, IL) or polyvinylidene difluoride membranes (Immun-Blot; Bio-Rad). Equal loading of the proteins was confirmed by Ponceau S staining. Immunoblotting analysis and densitometry were performed as previously described (55). Antibodies for signal transducer and activator of transcription 1 (STAT1) and phospho-STAT1 (Tyr701) were purchased from Cell Signaling (Beverly, MA); phospho-STAT2 antibody (Tyr689) was from Upstate Biotechnology (Lake Placid, NY); poly(ADP-ribose) polymerase (PARP) and inducible nitric oxide synthase (NOS2) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture, flow cytometry, and enzyme-linked immunosorbent assays (ELISAs). Single-cell suspensions of splenocytes were prepared from control and adenovirus-infected mice by squeezing spleens between sterile frosted slides; passing cell suspensions through 70-μm cell strainers; and lysing red blood cells in ammonium, potassium, and chloride buffer. Liver single-cell suspensions were obtained by passing pooled livers through sterilized 200-μm stainless steel mesh. Liver mononuclear cells were obtained by gradient centrifugation, and an excess of red blood cells was removed by lysis in ammonium, potassium, and chloride buffer. Relative percentages of $CD4^+$, $CD8^+$, and TCR $V\beta8^+$ T cells in spleen and liver cell isolates were determined by flow cytometry. *R*-phycoerythrin (PE)-labeled and fluorescein isothiocyanate (FITC)-labeled specific antibodies and isotype controls for flow cytometry were purchased from BD Biosciences (San Diego, CA). These were anti-CD4 (clone RM4-5), CD8 (clone 53-6.7), IFN-γ (clone XMG1.2), TCR Vβ8 (clone F23.1, which reacts with TCR Vβ8.1, 8.2, and 8.3), and TCR Vβ (clone H57-597, which reacts with a common epitope of all β chains of TCR). A total of 20,000 cells that satisfied a gate on forward and side scatter to eliminate aggregates and debris were evaluated using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) at the University of Iowa Flow Cytometry Facility. Data analysis was performed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

For cytokine production assays, the cells were cultured at 1.25×10^6 cells/ml in 24-well plates in RPMI 1640 medium supplemented with 10% fetal calf serum, nonessential amino acids, 2 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol (Sigma), and 50 μg/ml gentamicin (Mediatech, Herndon, VA). In some experiments, $CD4^+$ or $CD8^+$ cells were depleted using Dynabeads (Dynal Biotech Inc., Brown Deer, WI). Cell-free culture supernatants were collected after 24 h of stimulation with 10 μg/ml of SEB. DuoSet ELISA development kits (R&D Systems, Minneapolis, MN) were used to measure IFN-γ, IL-2, IL-4, and IL-6 following the manufacturer's recommendations. The mouse IFN-γ immunoassay kit (Biosource, Camarillo, CA) was also used in some experiments to increase the sensitivity of detection. Intracellular IFN-γ in $CD4^+$ or $CD8^+$ cells was also detected by two-color flow cytometry analysis. Following in vitro stimulation with SEB for 4 or 20 h, cells were treated with GolgiPlug (BD Biosciences) for 4 h, surface stained with FITC-labeled anti-CD4 or CD8 antibodies, fixed, and permeabilized and stained with PE-labeled anti-IFN-γ antibody. Unlabeled anti-IFN-γ antibody or recombinant mouse IFN-γ (R&D Systems) was added to negative controls prior to PE-labeled anti-IFN-γ to establish the threshold for IFN-γ-positive cells.

Caspase activity, TUNEL, and ALT assays for analysis of liver injury. Caspase activity in individual mouse liver lysates was measured in 96-well plates by diluting lysates in caspase assay buffer {20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2 } and incubating them with 50 μM of fluorogenic substrate Ac-DEVD-AMC (Calbiochem) at 37°C in the dark for 1 h. Fluorescence intensity was measured using a 355-nm excitation filter and a 460-nm emission filter in a Victor^2 microplate reader (EG & G Wallac, Gaithersburg, MD). Background fluorescence (substrate only) was subtracted from all wells; the fluorescence intensity was normalized to protein concentration, and the results were expressed as relative light units per μg protein (RLU/μg). The TdT-mediated dUTP nick end labeling (TUNEL) assay for apoptosis was performed on liver cryosections using the In Situ Cell Death Detection kit (TMR

Red; Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's recommendations as previously described (56). Alanine transaminase (ALT) activity in the serum samples was measured in the microplate reader using Infinity ALT reagent (ThermoELECTRON, Milford, MA).

Statistical analyses. The data were expressed as means ± standard errors of the means (SEM). The differences between control and adenovirus-infected groups in body temperatures, cytokine levels, and ALT activity were analyzed using an unpaired, two-tailed *t* test assuming equal variances. The effects of SEB treatment on caspase activity in the liver lysates in control and adenovirus-infected mice (total of four groups) were evaluated by analysis of variance. The selected groups were compared using the Bonferroni postanalysis test. The same approach was used to analyze differences in body temperatures for control and adenovirus-infected BALB/c and GKO mice (total of four groups) and for densitometry data from immunoblot analyses. All calculations were performed with GraphPad Prism software version 3.0 (GraphPad Software, San Diego, CA).

RESULTS

Adenoviral infection increases severity of responses to SEB. To evaluate the effects of prior exposure to adenovirus on responses to SEB, we used a model of systemic adenoviral infection in DBA/2 mice (*H-2*^{d} haplotype) with 10^6 PFU/mouse of wild-type human adenovirus 5 administered via intravenous route. We have previously shown that this regimen leads to tolerance to TNF-α and protects mice from SEB in the D-Gal model (56). Sublethal doses of SEB (125 to 250 μg/mouse) without D-Gal were administered intraperitoneally to control and adenovirus-infected mice on day 7 postinfection. We established in a pilot experiment that these doses of SEB were tolerated by control DBA/2 mice (data not shown). To compare the responsiveness of control and adenovirus-infected mice to SEB, we monitored mice for the signs of distress for 24 h after SEB treatment. In addition, we regularly measured body temperature, since a previous study has shown that the degree of hypothermia correlates with the morbidity and mortality from shock induced by treatment with staphylococcal enterotoxins and lipopolysaccharide (47). Previous studies have shown that noninvasive use of an infrared thermometer provides a reliable method of temperature measurement with minimal stress for animals (40). We observed that adenovirus-infected mice had significantly more profound hypothermia than control mice at 4 h after treatment with 125 μg/mouse of SEB (Fig. 1a). Some variations in body temperature of control mice were recorded (which apparently reflect the diurnal cycle), but the mean body temperature was always above 35.8°C (data not shown). SEB treatment of control and adenovirus-infected mice resulted in transient signs of distress such as piloerection, decreased mobility, and hunched stature. The adenovirus-infected group demonstrated more profound effects (data not shown). However, both groups of mice completely recovered by 24 h. Similar observations were made when 250 μg/mouse of SEB was used (data not shown). Thus, adenoviral infection increases severity of distress but does not increase mortality after treatment with SEB at the doses used in this study.

Increased susceptibility of influenza virus-infected mice to SEB is mediated by increased production of IFN-γ and TNF-α (57). As we have previously shown that adenoviral infection protects mice against the hepatotoxic effects of TNF-α (56), we focused our attention on the production of IFN-γ. We attempted to measure serum levels of IFN-γ on day 7 following adenoviral infection alone, as previous studies have shown induction of IFN-γ mRNA in the liver and secretion of IFN-γ by splenocytes following adenoviral infection (36, 54). We could not detect any IFN-γ in the serum of control or adenovirus-infected mice even when we used a high-sensitivity immunoassay (limit of detection is 2 pg/ml). IFN-γ production could be detected in cultures of unstimulated splenocytes from adenovirus-infected mice (up to 114 ± 5 pg/ml) but not from control mice. These data suggest that adenoviral infection alone induces production of IFN-γ; however, the production levels are too low to detect any IFN-γ in the serum.

SEB treatment of control and adenovirus-infected mice induced IFN-γ in the serum of both groups (Fig. 1b). However, the levels of IFN-γ were ninefold higher in the adenovirus-infected mice than in control mice at 4 h (peak levels) and sevenfold higher at 8 h (Fig. 1b). To determine whether this observation was specific for IFN-γ, we also measured serum IL-2 and IL-6. Prior to SEB exposure, these cytokines were undetectable in the serum of control or adenovirus-infected mice (data not shown). Peak levels of serum IL-2 following SEB exposure were 1.35 ± 0.24 ng/ml in control mice and 1.52 ± 0.33 ng/ml in adenovirus-infected mice (mean ± SEM, $n = 5$, no significant difference). Peak levels of serum IL-6 following SEB exposure were 9.03 ± 3.44 ng/ml in control mice and 10.41 ± 4.79 ng/ml in adenovirus-infected mice (mean ± SEM, $n = 5$, no significant difference). Thus, adenoviral infection primes mice for increased production of IFN-γ after SEB exposure but does not alter production of IL-2 or IL-6.

To determine whether IFN-γ is necessary for adenovirus-induced sensitization to SEB, we used IFN-γ-knockout mice (GKO) backcrossed to the BALB/c background. We infected GKO and wild-type BALB/c mice with human adenovirus as described above. Adenovirus alone had no significant effects on body temperature, as demonstrated at time zero (body temperature measured prior to SEB challenge). Control and adenovirus-infected mice of both strains were challenged with 125 μg/mouse of SEB in parallel in the same experiment (Fig. 1c). Adenoviral infection of BALB/c mice sensitized them to SEB, as they had significantly lower body temperature from 2 to 8 h following SEB treatment (compared to other groups at the same time points). In contrast, body temperature in adenovirus-infected GKO mice was not different from that of uninfected GKO or BALB/c mice at the same time points after SEB treatment. This suggests that IFN-γ is necessary for adenovirus-induced sensitization to SEB.

Adenoviral infection leads to splenomegaly and increases the absolute numbers of Vβ8$^+$ SEB-responsive T cells. In the next experiments, we attempted to determine the reason for excessive production of IFN-γ in response to SEB by adenovirus-infected mice. A number of previous reports have demonstrated that adenoviral infection induces a robust T-cell-mediated immune response involving extensive proliferation of adenovirus-specific CD4$^+$ and CD8$^+$ effector T cells (34, 50, 52, 53). We found that adenoviral infection for 7 days led to splenomegaly (Fig. 2a). The average weight of the spleens was significantly increased in adenovirus-infected mice (0.181 ± 0.028 g in adenovirus-infected versus 0.097 ± 0.014 g in control mice, mean ± SEM, $P < 0.05$, $n = 4$ mice). Spleen cellularity was also increased in adenovirus-infected mice, as evidenced by increased average cell recovery ([9.92 ± 2.07] × 10^7 cells/spleen in adenovirus-infected versus [6.18 ± 0.89] × 10^7 cells/

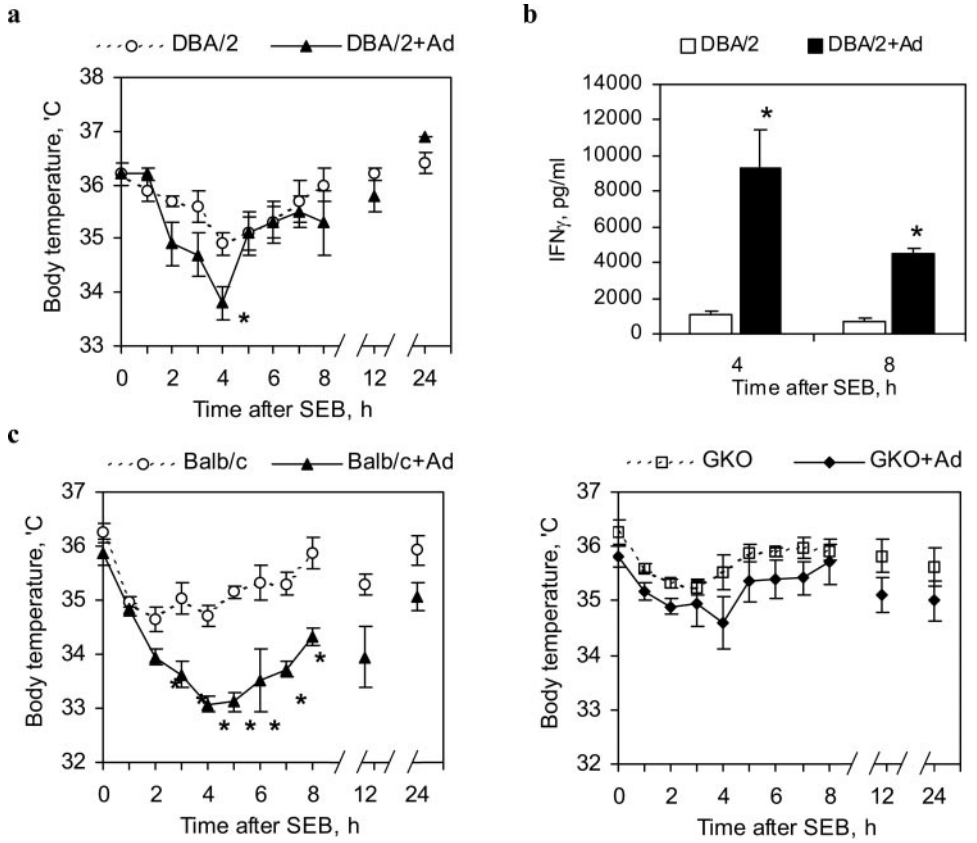

FIG. 1. Adenoviral infection increases severity of responses to SEB. (a) DBA/2 mice were infected with human adenovirus (serotype 5, 10^6 PFU/mouse) via the intravenous route for 7 days. Control and adenovirus-infected mice were challenged with 125 μg/mouse of SEB. Body temperature was measured using an infrared veterinary thermometer. The hypothermia was significantly more profound in adenovirus-infected mice than in control mice at 4 h after SEB treatment (marked with *, $P < 0.01$, $n = 13$). (b) Control and adenovirus-infected DBA/2 mice were euthanized at 4 h and 8 h after SEB exposure to collect blood samples. Serum cytokines were measured with ELISA. IFN-γ was significantly higher in adenovirus-infected mice than in control mice at 4 and 8 h after SEB treatment (*, compared to the controls at the same time points, $P < 0.01$, $n = 4$). (c) IFN-γ-deficient mice (GKO) on a BALB/c background and wild-type BALB/c mice were infected with human adenovirus (serotype 5, 10^6 PFU/mouse) via the intravenous route for 7 days. Control and adenovirus-infected mice were challenged with 125 μg/mouse of SEB. Adenovirus-infected BALB/c mice had significantly lower body temperatures from 2 to 8 h after SEB treatment than did other groups at the same time points (marked with *, $P < 0.05$, $n = 4$ mice in each group). The kinetics of body temperature is shown in two panels for clarity, but all groups were treated in parallel in the same experiment.

spleen in control mice, mean ± SEM, $P < 0.05$, $n = 4$ experiments with three to four mice in each experiment). SEB has preferential affinity for the Vβ3, -7, -8.1 to -8.3, and -17 chains of TCR (29). Therefore, it was possible to suggest that adenovirus-induced priming for IFN-γ production in response to SEB could be explained by increased numbers of SEB-responsive T cells. The most abundant group of SEB-responsive T cells in DBA/2 mice express Vβ8.2 and Vβ8.3 TCR chains. T cells expressing Vβ8.1 are deleted in the thymus due to their reactivity to the minor lymphocyte-stimulating antigen Mls-1^a, and T cells expressing other Vβ chains are rather scarce (22, 29). Therefore, we analyzed the relative abundance of SEB-responsive T cells in the single-cell suspensions from the spleens of control or adenovirus-infected mice using a monoclonal antibody against Vβ8 chains. We found that adenoviral infection has little if any effect on the relative numbers of T cells expressing the TCR Vβ8 chain (Fig. 2b). Staining with an antibody detecting all TCR Vβ chains also showed that the relative numbers of $CD4^+$ or $CD8^+$ T cells were minimally affected (data not shown). Taken together, these data indicate that adenoviral infection increases the absolute numbers of SEB-responsive T cells in the spleen but does not influence the proportion of SEB-responsive T cells.

Intravenous administration of adenovirus preferentially targets the liver and leads to liver infiltration with virus-specific lymphocytes (51, 52). To determine whether adenoviral infection alters the number of SEB-responsive T cells in the liver, we isolated liver mononuclear cells from control and adenovirus-infected mice and determined the relative number of cells expressing the TCR Vβ8 chain using flow cytometry. We found that the number of SEB-responsive T cells in the liver increased more than twofold following adenoviral infection, primarily due to accumulation of $CD8^+$ T cells (Fig. 2c and data not shown).

Splenocytes from adenovirus-infected cells produce increased amounts of IFN-γ early after stimulation with SEB in vitro. We next determined whether adenoviral infection primes for early increased production of IFN-γ by liver mononuclear

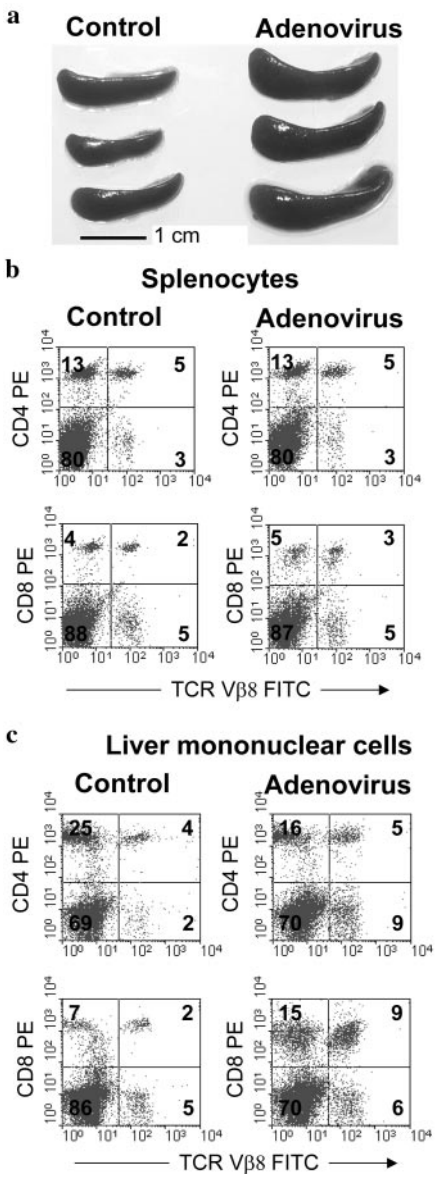

FIG. 2. Effects of adenoviral infection on T cells in spleen and liver. (a) Spleens from control and adenovirus-infected DBA/2 mice are shown. (b) Flow cytometry analysis of SEB-responsive T cells in the splenocyte populations. Splenocytes from control and adenovirus-infected mice were analyzed after staining with antibodies recognizing Vβ8 TCR (FITC labeled) and CD4 or CD8 (PE labeled). The data are representative of three independent experiments. (c) Flow cytometry analysis of liver mononuclear cells isolated from control and adenovirus-infected mice was performed as in panel b.

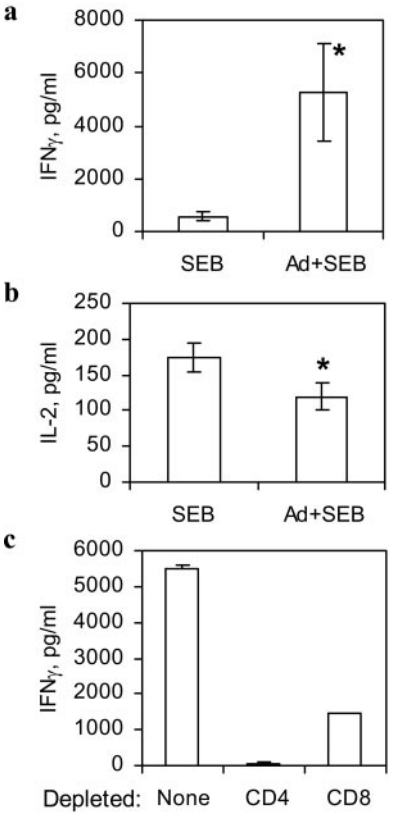

FIG. 3. Production of IFN-γ and IL-2 in splenocyte cultures after in vitro stimulation with SEB. Splenocytes from control and adenovirus-infected mice were stimulated with SEB for 24 h. Cytokines were measured by ELISA. (a) Splenocytes from adenovirus-infected mice produce significantly higher amounts of IFN-γ after 24 h of stimulation with SEB (*, $P < 0.05$; the data are means ± SEM from seven independent experiments). (b) Splenocytes from adenovirus-infected mice produce moderately lower amounts of IL-2 after 24 h of stimulation with SEB (*, $P < 0.01$; the data are means ± SEM from seven independent experiments). (c) Depletion of $CD4^+$ T cells with magnetic beads prior to stimulation with SEB eliminates IFN-γ production by splenocytes from adenovirus-infected mice. Depletion of $CD8^+$ T cells also decreases the levels of IFN-γ. Representative data from two independent experiments are shown (means ± SEM from triplicate cultures).

cells and splenocytes in vitro. This was done to determine whether increased IFN-γ levels after SEB treatment are due to increased cellularity and absolute numbers of SEB-responsive T cells or due to increased production of IFN-γ per cell. Liver mononuclear cells and splenocytes from control and adenovirus-infected mice were stimulated with the optimal dose of SEB (10 μg/ml). The levels of IFN-γ, IL-2, and IL-4 in the culture supernatants were measured at 24 h poststimulation. The levels of IFN-γ in the cultures of liver mononuclear cells were very low and not different between control and adenovirus-infected groups (data not shown). This suggests that adenoviral infection does not prime liver-infiltrating T cells for increased production of IFN-γ per cell after SEB stimulation. However, we found that splenocytes from adenovirus-infected mice produced ninefold more IFN-γ than the control splenocytes by 24 h poststimulation (Fig. 3a). The levels of IL-4 were barely above the detection limit at 24 h poststimulation and comparable between the groups (data not shown). The production of IL-2 was moderately, but significantly, lower in the cultures from adenovirus-infected mice (Fig. 3b). These data demonstrate that not only are there increased numbers of SEB-reactive T cells after adenoviral infection but there is also increased IFN-γ production by splenocytes on a per-cell basis.

$CD4^+$ and $CD8^+$ T cells produce IFN-γ in response to SEB (14). We next determined whether $CD4^+$ or $CD8^+$ T cells from adenovirus-infected mice are the major source of IFN-γ after SEB treatment. We used intracellular cytokine staining

and two-color flow cytometry to determine production of IFN-γ by $CD4^+$ and $CD8^+$ cells. Early production of IFN-γ (within 8 h after in vitro stimulation with SEB) was found in a very small fraction of the cells (less than 1%) isolated from adenovirus-infected mice but not from control mice (data not shown). IFN-γ-positive cells were found within $CD4^+$ or $CD8^+$ cells (data not shown). Prolonged stimulation increased the number of IFN-γ-positive cells, but the difference between control and adenovirus groups in the number of IFN-γ-positive cells disappeared (data not shown). Furthermore, we depleted either $CD4^+$ or $CD8^+$ T cells using magnetic beads prior to stimulation with SEB and measured IFN-γ production by ELISA. We found that depletion of $CD4^+$ T cells effectively wiped out IFN-γ production in the SEB-stimulated cultures (Fig. 3c). However, depletion of $CD8^+$ T cells also decreased production of IFN-γ relative to parallel undepleted cultures. Taken together, these data suggest that IFN-γ is derived from both $CD4^+$ and $CD8^+$ T cells.

We noted that the levels of IFN-γ in CD4- or CD8-depleted cultures did not add up to the levels of IFN-γ in the control undepleted cultures. The possible explanation is that a large proportion of splenic dendritic cells are known to express either CD4 or CD8 (44). These cells are likely to be important for stimulation of T cells by SEB and may have been depleted by the anti-CD4 or anti-CD8 magnetic beads. We have also tested a possibility that $CD4^+$ $CD8^+$ double-positive cells may appear in the spleens of adenovirus-infected mice, as these cells were previously described in the lymph nodes of mice infected with adenoviral vectors (15). However, we did not observe double-positive T cells in the spleens in our model (data not shown).

IFN-γ signaling after SEB exposure is more profound in adenovirus-infected mice. Responses to IFN-γ are mediated by tyrosine phosphorylation of STAT1, whereas responses to type I interferons (IFN-α and IFN-β) are mediated by tyrosine phosphorylation of both STAT1 and STAT2 (18). It has been previously shown that IFN-γ-induced STAT1 phosphorylation plays a detrimental role in T-cell-mediated hepatitis in mice treated with concanavalin A (16, 19). To determine the effects of increased levels of IFN-γ in adenovirus-infected mice after SEB exposure, we used immunoblot analysis to detect tyrosine phosphorylation of STAT1 and STAT2. Prior to SEB exposure, STAT1 phosphorylation was very low in control and adenovirus-infected mice. Treatment of control mice with SEB induced STAT1 phosphorylation. Significantly higher levels of STAT1 phosphorylation were observed in mice that were infected with adenovirus and treated with SEB. We also noticed that the total levels of STAT1 in the liver were increased in adenovirus-infected mice (Fig. 4b). SEB treatment had no effects on total STAT1 expression. Phosphorylation of STAT2 was not detected in any of the groups (data not shown). This suggested that STAT1 phosphorylation in adenovirus-infected mice is induced by IFN-γ.

Next, we evaluated expression of NOS2 to determine whether increased STAT1 phosphorylation was physiologically significant for mice exposed to adenovirus and SEB. A previous study has shown that expression of NOS2 is positively regulated by IFN-γ (10). We found that expression of NOS2 was high in the mice exposed to adenovirus and SEB but could not be detected in any other group (Fig. 4c).

To clarify the relationship between IFN-γ and STAT1 phosphorylation and NOS2 expression, we performed immunoblot analyses of lysates from GKO and BALB/c mice (Fig. 4d). BALB/c mice demonstrated the same effects of adenoviral infection and SEB treatment as DBA/2 mice. In contrast, GKO mice showed no STAT1 phosphorylation after adenovirus or SEB treatment. This further indicated that STAT1 phosphorylation following SEB treatment is dependent on IFN-γ. The effects of adenoviral infection on expression of total STAT1 were attenuated in GKO mice. This suggested that increased expression of STAT1 following adenoviral infection is only partially dependent on IFN-γ. Expression of NOS2 was induced in BALB/c mice infected with adenovirus and treated with SEB but not in other groups. The same treatment of GKO mice did not result in induction of any NOS2. Therefore, SEB treatment of adenovirus-infected mice results in increased phosphorylation of STAT1 and expression of NOS2 via an IFN-γ-dependent mechanism.

Adenoviral infection sensitizes mice to liver injury following SEB treatment. Previous studies have shown that IFN-γ may trigger apoptosis in hepatocytes in vitro and in vivo (16, 21). To determine whether SEB treatment leads to apoptosis in the liver of adenovirus-infected mice, we measured activity of caspases in the liver lysates using a fluorogenic substrate, which can be cleaved by caspase 3 or 7. Very low caspase activity was found in the liver lysates of control mice prior to and after SEB treatment (Fig. 5a). Adenoviral infection itself, without SEB exposure, showed a trend to increase caspase 3 activity, but the difference was not statistically significant. However, SEB treatment increased caspase activity in the livers of adenovirus-infected mice to the extent that it became significantly higher than in the livers of control mice (Fig. 5a).

To determine whether increased caspase activity in the liver was physiologically important, we performed an immunoblot analysis of liver lysates for PARP, which is known to be cleaved by caspase 3 or 7 in vivo during apoptosis (46). We found no evidence of PARP cleavage in the livers of control mice with or without SEB stimulation (Fig. 5b). Adenovirus-infected mice showed virtually undetectable PARP cleavage. However, cleaved PARP was readily detectable in the livers of adenovirus-infected mice after SEB exposure (Fig. 5b).

Next, we determined whether SEB treatment of adenovirus-infected mice led to destruction of hepatocytes. We measured serum levels of ALT, a liver-specific enzyme, which is routinely used as a marker of liver injury (16, 25, 26). Prior to SEB treatment, adenovirus-infected mice had slightly higher ALT levels than control mice, albeit the difference was not significant (Fig. 6a). Following SEB treatment, serum ALT levels were significantly higher in adenovirus-infected mice than in control mice. These data indicate that SEB-induced liver injury is more evident in adenovirus-infected than in control mice.

Analysis of liver sections by TUNEL assay confirmed increased apoptosis in the liver of adenovirus-infected mice after SEB exposure. We observed very little apoptosis in the liver sections from control and adenovirus-infected mice without SEB exposure (data not shown). SEB treatment of control mice did not increase the extent of apoptosis (Fig. 6b, top panel). This is consistent with the previous study showing that SEB alone, without D-Gal sensitization, does not have hepatotoxic effects (1). However, there were more apoptotic hepa-

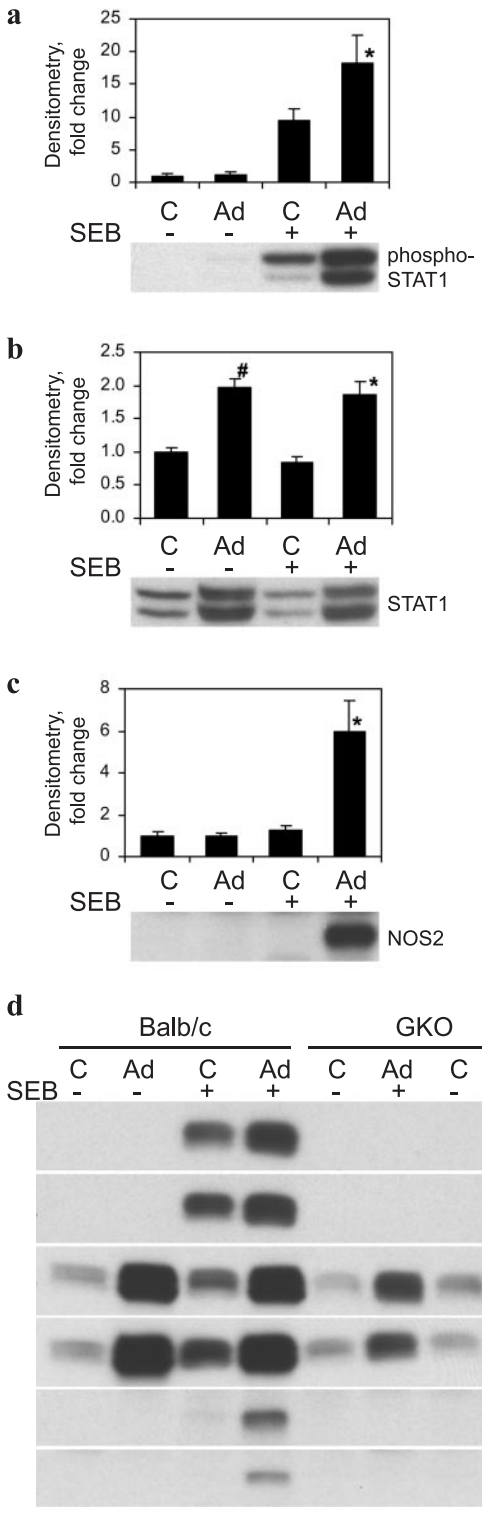

FIG. 4. Increased IFN-γ signaling in the livers of adenovirus-infected mice following SEB exposure. Phosphorylation of STAT1 (a) and total levels of STAT1 (b) and NOS2 (c) at 4 h after SEB treatment of DBA/2 mice were evaluated by immunoblot analysis of liver lysates. Densitometry data (means ± SEM of four individual mice per group) are shown. The signal intensities of phospho-STAT1, total STAT1, and NOS2 were significantly higher in the group treated with adenovirus and SEB (*, $P < 0.05$, compared to the group treated with SEB alone). The signal intensity of total STAT1 was significantly higher in the adenovirus-infected group (#, $P < 0.01$, compared to control group). Representative scans are shown below each densitometry chart. (d) Phosphorylation of STAT1 and total levels of STAT1 and NOS2 at 4 h after SEB treatment of BALB/c and GKO mice were evaluated by immunoblot analyses. Two mice per group were used to perform immunoblot analyses in individual lysates and shown in two scans for each protein. Equal loading was confirmed by Ponceau S staining of the blots (not shown).

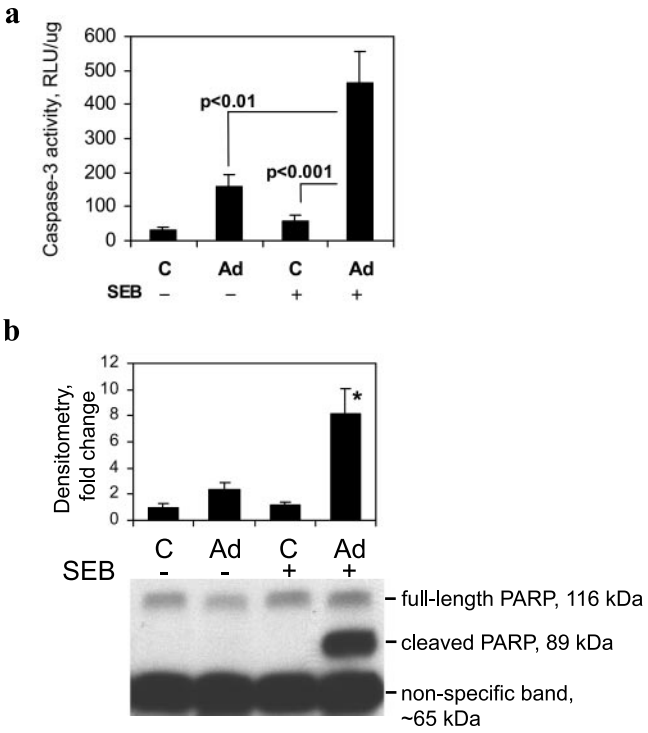

FIG. 5. SEB exposure increases caspase activity in the liver in adenovirus-infected DBA/2 mice. (a) Caspase activity in the liver lysates was measured using a fluorogenic substrate, Ac-DEVD-AMC, which can be cleaved by caspase 3 or 7. The fluorescence intensity was normalized to protein concentration (relative light units [RLU] per μg protein). Caspase activity was significantly increased in the livers of adenovirus-infected mice at 4 h after SEB treatment and was significantly higher than in the livers of mice treated with SEB alone ($n = 5$ mice). (b) Cleavage of PARP was evaluated by immunoblot analysis. Densitometry data (means ± SEM of four individual mice per group) are shown. A representative scan is shown. The signal intensity of cleaved PARP (89 kDa) was significantly higher in the group treated with adenovirus and SEB (*, $P < 0.01$, compared to the group treated with SEB alone). Equal loading was confirmed by Ponceau S staining of the blots (not shown).

tocytes in the liver sections from adenovirus-infected mice after SEB treatment (Fig. 6b, bottom panel). Thus, our data show that control mice have very little apoptosis in the liver after SEB exposure, whereas adenovirus-infected mice display increased apoptosis evidenced by increased caspase activity, cleaved PARP, and TUNEL staining.

To determine whether the effects of adenoviral infection on SEB exposure were confined to the liver, we also evaluated caspase activity and PARP cleavage in the lung lysates. Caspase activity was slightly higher in the lungs of adenovirus-infected mice than in those of control mice (data not shown). However, SEB treatment had no effect on caspase activity and

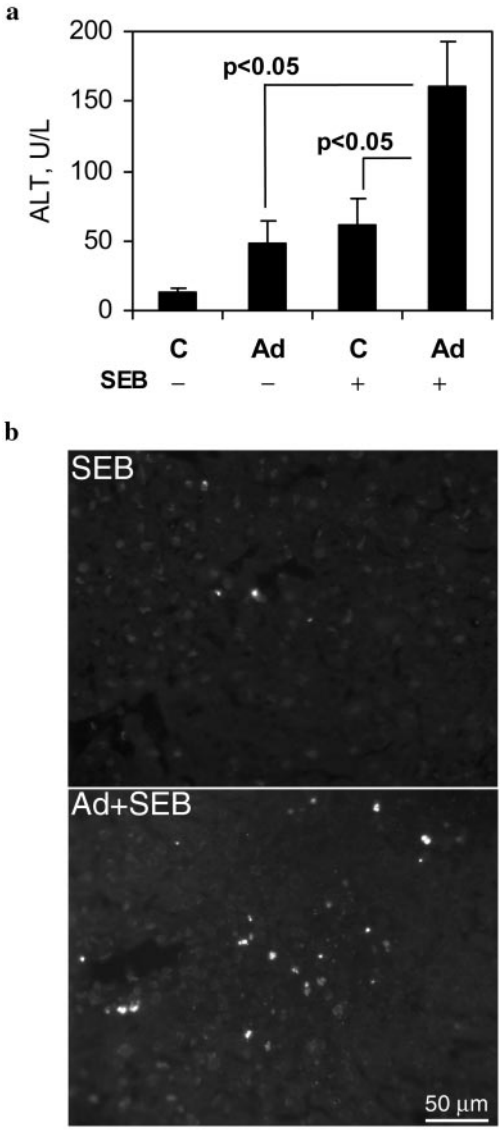

FIG. 6. Adenoviral infection sensitizes mice to liver injury induced by SEB. (a) Serum samples were obtained from control (C) and adenovirus-infected (Ad) mice exposed to SEB for 24 h or left untreated. Serum levels of ALT (expressed in specific units per liter [U/L]) significantly increased in adenovirus-infected mice at 24 h after SEB and were significantly higher than in mice treated with SEB alone ($n = 4$). (b) TUNEL assay for apoptosis in the liver of control and adenovirus-infected mice at 8 h after SEB exposure. Cryosections were prepared and analyzed from four mice from each group. Representative photomicrographs are shown.

we could not detect PARP cleavage in any group (data not shown).

DISCUSSION

In the present study, we characterized the effects of adenoviral infection on the responses to SEB without D-Gal sensitization. Exposure to SEB alone in the absence of infection caused minimal liver injury, consistent with a previous report (1). We found that systemic adenoviral infection increases the severity of liver injury following exposure to SEB. Treatment of adenovirus-infected mice resulted in more profound hypothermia (Fig. 1a), increased apoptosis in the liver, and higher levels of ALT in the serum (Fig. 5 and 6). Adenovirus-infected IFN-γ-deficient mice on a BALB/c background showed no hypothermia following SEB exposure, whereas their wild-type counterparts (BALB/c mice) became sensitive to SEB following adenoviral infection (Fig. 1c). This established the requirement for IFN-γ in adenovirus-induced sensitization to SEB. Significantly, adenoviral infection increased the absolute number of T cells in the spleen and primed $CD4^+$ T cells for early IFN-γ production in response to SEB treatment (Fig. 3).

It is important to note that the chosen regimen of adenoviral infection caused minimal liver injury prior to SEB exposure. Previous studies have shown that administration of high doses (10^9 to 10^{11} PFU/mouse) of E1,E3-deleted adenoviral vectors leads to significant liver injury (8, 25, 52). We used wild-type adenovirus with the genes of immune evasion preserved and at a relatively low dose (10^6 PFU/mouse) to minimize liver injury from adenovirus infection itself. Adenovirus-infected mice had slightly elevated, albeit not significantly, caspase activity (Fig. 5a) and serum ALT levels (Fig. 6b) on day 7 postinfection. However, we found no significant evidence of PARP cleavage (Fig. 5b) and very low levels of apoptosis using TUNEL staining in the livers of adenovirus-infected mice prior to SEB exposure.

IFN-γ has previously been characterized as a key cytokine in SEB-induced toxic shock and virus-induced sensitization to SEB (30, 57). Adenoviral infection is known to induce robust T-cell-mediated immune responses accompanied by preferential production of Th1 cytokines (17, 35, 51). We found that adenoviral infection primed for excessive production of IFN-γ after stimulation with SEB in vivo (Fig. 1b) and in vitro (Fig. 3a).

Our observation of increased sensitivity to SEB following adenoviral infection has some similarities to the previous study that demonstrated lethal synergism between influenza virus infection and SEB (57). In both cases, increased levels of IFN-γ were observed in the infected mice. However, adenovirus-infected mice were able to recover from the doses of SEB that were lethal to the influenza-infected mice. The critical role of both IFN-γ and TNF-α in sensitization to SEB following influenza infection has been postulated, as neutralization of either cytokine showed a significant protective effect (57). It is possible that adenovirus-induced tolerance to TNF-α, which we described in our previous paper (56), limits the extent of sensitivity to SEB in adenovirus-infected mice. Another explanation for the difference could be based on the fact that the infection in the lungs was critical for sensitization to SEB after influenza virus (57), whereas we did not observe any involvement of the lungs in our model.

We took our investigation further and showed for the first time increased IFN-γ signaling and apoptosis in the liver of adenovirus-infected mice after SEB exposure (Fig. 4 to 6). IFN-γ plays a key role in concanavalin A-induced hepatitis, which is also mediated by hyperactivation of liver T cells and NKT cells (16, 19). IFN-γ is known to increase expression of Fas and to promote killing of hepatocytes by cytotoxic lymphocytes (37). In addition, IFN-γ is known to induce hepatocyte apoptosis in vitro via induction of IRF-1 (21). On the other

hand, adenovirus has been previously shown to block IFN-γ signaling in vitro (20, 27). Therefore, it was important to determine whether increased IFN-γ following SEB exposure is physiologically relevant and results in increased biological responses in vivo. We found that exposure of adenovirus-infected mice to SEB resulted in significantly higher levels of phosphorylated STAT1 in the liver than in mice treated with SEB alone. Furthermore, NOS2 could be detected only in the group of mice that was exposed to both adenovirus and SEB. Phosphorylation of STAT1 and induction of NOS2 were apparently dependent on IFN-γ, as these events were not observed in interferon-deficient mice. This demonstrates that IFN-γ signaling, at least in the liver, is not inhibited by adenoviral infection. Thus, sensitization to SEB exposure following adenoviral infection is mediated, at least in part, by high levels of IFN-γ.

It has been previously shown that respiratory viral infections increase the risk of development of toxic shock syndrome mediated by bacterial superantigens (6, 28, 43, 45). Viral infections have been suggested to pave the way for secondary bacterial infections by inhibiting the immune system and facilitating bacterial colonization (13). Our data show that preexisting adenoviral infection significantly increases pathology after subsequent SEB exposure. Furthermore, adenovirus increases both the number of SEB-responsive T cells and IFN-γ production on a per-cell basis. Therefore, our study suggests that hyperresponsiveness of T lymphocytes at the peak of antiviral immune responses mediates increased morbidity when a bacterial superantigen follows a viral infection.

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